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Dynamics of the SNARE-mediated synaptic vesicle fusion pore

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

For my great-grandfather, Fred Steele.

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ABSTRACT

SNARE-mediated membrane fusion is a key step in the process of neurotransmission. The release of neurotransmitters into the synapse as a result of vesicle fusion, particularly when triggered by an influx of Ca^{2+}, serves as a primary means of signal propagation and communication in cognitive processes. Much work has been done to study these SNARE-mediated fusion events and the role auxiliary proteins play in modulating the overall process. However, our understanding of certain stages in the process is comparatively lacking, and our ability to study them has so far been curtailed by the relatively primitive state of current assays. This is particularly true for the opening and expansion of the fusion pore following hemifusion, necessary for the release of neurotransmitters from the vesicle into the synapse, which has gone largely unexamined despite its importance in understanding both the initial release and the life cycles of neurotransmitter-carrying vesicles.

Despite prior difficulties, refinement of an *in vitro* single vesicle fusion assay to a sufficiently robust state has presented an opportunity to investigate. In this study, we develop a novel method for the study of fusion pore dynamics. Utilizing vesicles containing fluorescently labeled dextran with a vesicle-to-suspended bilayer fusion assay, we can observe the release the release of the dextran through the bilayer as the pore opens and expands beyond the hydrodynamic radius of the dextran. We further observe a halt in release as the pore shrinks beneath this radius, giving rise to multi-staged content release patterns common to many vesicle docking and fusion events in the presence of SNAREs alone.

With this assay developed, we also perform preliminary studies into the roles of SNARE accessory proteins known to impact fusion. In particular, we observe the ability of α-synuclein

to influence fusion pore dilation. While still in its early stages, we believe that our single vesicle content release assay can provide valuable insight into the roles of this and other proteins in modulating the dynamics of the fusion pore, and a greater understanding of the mechanisms of SNARE-mediated fusion as a whole.

CHAPTER 1

INTRODUCTION

1.1 Neurotransmitter release in response to action potential

Tightly regulated interneuronal communication is necessary for a properly functioning brain. Rapid depolarization of the cell membrane beyond a certain threshold in response to a signal triggers an action potential which is carried down the cell, allowing an influx of positively-charged ions through voltage-gated channels (1, 2). As this action potential reaches the pre-synaptic terminal, docked and primed vesicles carrying neurotransmitters will synchronously fuse to the plasma membrane in response to the influx of Ca^{2+}, forming an opening and releasing their cargo into the synapse where they can be picked up by receptors on the post-synaptic terminal, thus propagating the signal through the nervous system (3, 4). This fusion process occurs in less than 1 ms, and the merged vesicles involved will be recycled and re-used in transmission of a later signal (4, 5, 6) (Figure 1).

It is generally agreed that vesicle-to-plasma membrane fusion is dependent upon soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (7, 8). However, despite being a major topic of study, many aspects of the SNARE-mediated fusion pathway remain poorly understood. The fusion pore is a major example of this, as an understanding of its dynamics has so far eluded researchers, and the involvement of protein factors other than SNAREs remains largely unknown (9, 10). Moreover, the notion that SNAREs alone are sufficient to determine how the pathway proceeds has increasingly come under scrutiny. Recent work has done much to show that several accessory proteins have key roles in many stages of

synaptic membrane fusion, and that the highly regulated fusion seen in normally functioning systems is dependent upon them and SNAREs working in concert (11-14).

1.2 Neuronal SNAREs mediate vesicle fusion

While SNAREs might not be sufficient to explain the highly regulated fusion observed during neurotransmission, they remain a necessary part of it. Absent the components of the complex, membrane fusion remains energetically unfavorable, and will not proceed (15). Current models accordingly hold that the SNARE complex remains the core molecular machinery driving membrane fusion (7, 8).

There are three SNAREs of particular interest in most studies of synaptic vesicle fusion, divided into vesicle (v-) and target (t-) categories depending on the membrane with which they are most commonly associated (16). The first of these is the v-SNARE vesicle associated membrane protein 2 (VAMP2), most commonly located on the vesicle membrane. Its counterparts on the target plasma membrane are the t-SNAREs syntaxin 1a and synaptosomal nerve-associated protein 25 (SNAP-25). Of the three, VAMP2 and syntaxin 1a (STX1A) are tail-anchored to their respective membranes, while SNAP-25 is a soluble protein typically attached to the target membrane by post-translational palmitoylation. Together, they are capable of assembling into the ternary SNARE complex (7, 17, 18, 19).

The SNARE complex is a stable coiled-coil, consisting of four alpha helices. These helices result from interactions between SNARE motifs, a conserved 60-70 amino acid long heptad repeat located in the cytosolic domain, common to all proteins in the SNARE family (20). Unlike STX1A and VAMP2, both of which have a single motif linked by its C-terminal end to the transmembrane domain, SNAP-25 has two such motifs connected by a loop (21). When

brought into contact, these motifs spontaneously transition from an unstructured state to the coiled-coil bundle, with SNAP-25 accordingly contributing two of the alpha helices and the other two SNARE proteins contributing one each (20, 22, 23) (Figure 2).

The hydrophobic core of the SNARE complex results from the interior positioning of the *a* and *d* residues of the heptad repeat. This results in a sixteen layer stack numbered -7 to +8 from N- to C-terminus, with a 0 layer composed of one arginine and three glutamine residues (24), leading to an alternative classification system often used for SNAREs to distinguish between arginine-contributing (R-SNARE) and glutamine-contributing (Q-SNARE) bodies. R-SNAREs and Q-SNAREs generally correspond to v- and t-SNAREs, respectively (16).

The initial stages of vesicle fusion are believed to be driven by SNARE 'zippering,' in which the *trans*-SNARE complex is formed from N- to C-terminal end. This first serves to draw the membranes closer together, priming them for fusion and leading to the formation of the fusion pore (15, 25). Accounts of how this occurs have historically differed. In the past, it has been suggested that the trans-membrane domains of SNAREs form a closed proteinaceous fusion pore after priming, which may then expand further to release content (26). However, the observation of lipid mixing prior to or without content release, and evidence that the fusion pore is primarily lipid-lined, present serious issues for the acceptance of this model (27, 28). Instead, the primary intermediate stage in vesicle fusion is believed to be *hemifusion*, a state in which the lipid membranes mix but have yet to fuse sufficiently for content release. Formation and dilation of the fusion pore follows after hemifusion occurs (29-34).

1.3 The fusion pore and vesicle recycling

The fusion pore is a channel formed by the fusion of the vesicle and presynaptic terminal membranes, exposing the interior of the vesicle to the synaptic cleft. When the pore is dilated to a sufficient size, this permits release of neurotransmitters into the synapse, and thus the propagation of neural signals (26, 35). It is widely believed that the formation of the *trans*-SNAREpin provides the energy necessary for both initial lipid merger in hemifusion and the opening of the fusion pore (20, 36). From here, however, there is significant disagreement, with two main mechanisms proposed.

The first is the full fusion mechanism. Here, the opened fusion pore will continue to dilate until the vesicle has fully collapsed into the plasma membrane, completely joining with it and releasing all its content in the process (Figure 3). In order to recycle this vesicle, it would need to be separated and retrieved from the plasma membrane, likely using clathrin-mediated endocytosis (37). However, pore dilation is predicted to be an extremely energy intensive process, and it is unclear whether formation of the *trans*-SNAREpin alone is sufficient to drive the process to full fusion (38, 39).

An alternative explanation is termed the 'kiss-and-run' mechanism. Here, rather than fully joining with the plasma membrane, the fusion pore only engages in dilation sufficient to release much of its content before contracting again. The fusion pore is then expected to eventually close, allowing the vesicle to retreat from the membrane after SNAREs disassociate. These vesicles could then be refilled and reused (40, 41).

The extent to which either the kiss-and-run or the full fusion mechanism plays a role in development of the fusion pore in neuronal SNARE-mediated vesicle fusion remains poorly

understood. Evidence suggests both may be involved (42). However, given the current poor understanding of fusion pore dynamics, it is difficult to say whether synaptic vesicles utilize one over the other, and under what conditions they might do so.

1.4 Accessory proteins

1.4.1 Alpha-synuclein

Accessory proteins have long been believed to influence SNARE-driven fusion. Many studies have been undertaken to understand which play a key part, and how the interaction of these proteins with SNAREs serves to regulate or drive the fusion process. The results of these studies paint an increasingly complex picture of the complex and reinforce the notion that SNAREs alone are necessary-but-insufficient for explaining fast, regulated fusion as observed *in vivo* (43). Even so, the roles and mechanisms of many of these proteins remain mysterious. This is particularly true for alpha-synuclein.

Alpha-synuclein (αS) is a soluble, 14 kDa protein, best known for its clinical significance in neurological diseases like Parkinson's disease and Lewy body dementia. Under pathological conditions, it aggregates into the insoluble neuronal plaque and Lewy bodies characteristic of such diseases (44, 45, 46), and it has been shown to directly inhibit SNARE-mediated membrane fusion when present under similar circumstances in excessively high concentrations (47, 48) (Figure 4). This property gave rise to the name for its central non-amyloid-β component (NAC) peptide region, for its presence in Alzheimer's disease amyloid (49). However, the non-pathological function of αS has proven far more elusive.

Even in healthy specimens, αS is found in abundance in the brain. It has a particularly high concentration in the presynaptic terminal of neurons, indicating it may be involved in the

regulation of neurotransmission (50, 51). Past evidence has shown that it is likely involved in some stages of vesicle recycling (51, 52, 53), and recent studies suggest an ability to enhance vesicle docking and promote formation of the SNARE complex itself through binding with VAMP2 via its unstructured C-terminal region. Its ability to accomplish this without reducing SNARE activity at increasingly high concentrations when not aggregating is particularly notable in light of past work with its pathological form, lending further credence to the idea of its importance in normal function (13, 54, 55).

The protein is also notable for its ability to interact with and distort membrane bilayers, having a high affinity for membrane curvature and negatively charged lipids, and is induced to form an α-helical structure at its N-terminal end on binding (56, 57, 58). This is certainly of note in terms of prior experiments with docking, but it also raises a question as to whether the protein might play further roles in the fusion process. It is possible that the ability of αS to distort bilayers serves to reduce the energy requirement of fusion pore dilation, or otherwise play an important role in promoting pore dilation and neurotransmitter release, and whether it might do so while also helping draw the vesicle close enough to the plasma membrane for SNAREs to initiate the opening of the fusion pore. While some studies suggest this may indeed be the case (59), others have proven inconclusive, suggesting additional research is necessary to determine whether this is the case.

1.4.2 Synaptotagmin 1

By contrast, synaptotagmin 1 (Syt1) is a SNARE-accessory protein bound to the synaptic vesicle by a helical, N-terminal transmembrane domain. Syt is of particular note for its cytosolic domains, consisting of a linker region, the C2A domain, and the C2B domain. These C2 domains, capable of binding as many as 5 Ca^{2+} ions at a time, have long been of interest to

researchers exploring the Ca^{2+} sensitivity of SNARE-mediated vesicle fusion (Figure 5). This interest was further reinforced by the capacity of Syt1 to bind to SNAREs, and the lack of observed Ca^{2+} sensitivity in the absence of Ca^{2+}-binding Syt1 *in vivo* strongly implicates it as the Ca^{2+} sensor in synaptic vesicle fusion (11, 60, 61).

As more evidence has emerged, the conception of Syt1 as the Ca^{2+} sensor in synaptic vesicle fusion appears increasingly well-founded. Recent *in vitro* experiments involving Syt1 in the presence of Ca^{2+} have been shown to markedly increase both hemifusion and the release of vesicle cargo through a fusion pore (62, 63). However, this does come with several caveats. Notably, these include the outright inhibition of SNARE-mediated fusion in the presence of Syt1, if the Syt1 is allowed to interact with the SNAREs prior to the injection of Ca^{2+} into the system (62, 64, 65).

It also raises the question of how, precisely, Syt1 in the presence of Ca^{2+} serves to promote fusion. In particular, the impact of Syt1 on the dynamics of the synaptic vesicle fusion pore after pore opening remain poorly understood. Given the known ability of Syt1 to bind to curved membranes, and the demonstrated ability of the C2B domain to cross-bridge membranes, it is entirely possible that the protein could serve to stabilize the fusion pore and possibly permit further expansion (66, 67, 68). While recent experiments indicate that it is likely Syt1 increases fusion pore dilation, the limitations of current *in vitro* assays make the question difficult to examine and the extent difficult to confirm (66, 69).

1.5 Single molecule assays for study of SNAREs

While bulk *in vitro* assays are still widely applied, their low resolution prevents researchers from precisely probing swift, dynamic systems of interest, and limits their ability to

unravel and understand individual steps in complicated processes. As such, single molecule assays have seen increasing use in the study of biomolecular machinery (70). Many unique techniques have been developed and refined over the past decade, opening the door to a deeper understanding of many diverse biochemical topics.

One such technique, single molecule total internal reflection fluorescence (smTIRF) microscopy, has proven particularly attractive for the study of neuronal SNAREs. In this system, incident light is transmitted through two materials with differing refractive indices. The light is adjusted such that the incident angle reaches the critical angle, at which it is refracted parallel to the surface of the material interface. An exponentially-decaying evanescent field ~200 nm in diameter generated by the reflection of the light can then be used to excite fluorophores without deep penetration, dramatically reducing noise and permitting observation of individual events within a very small range close to the boundary of the mediums (71) (Figure 6). All this can be done on a millisecond timescale and, in principle, can be used to perform detailed observations of the dynamics of all stages in vesicle fusion, avoiding some of the temporal or content limitations seen with other single vesicle techniques (72).

Purified SNAREs have been shown to reconstitute into liposomes, permitting the synthesis of v- or t- vesicles and providing researchers a valuable tool for *in vitro* membrane fusion experiments. This has previously been used to great effect with the bulk lipid mixing assay. By labeling lipids on t- and v- vesicles with FRET-paired dyes, increases in intensity over time could be used to monitor rates of lipid mixing and determine the impact of SNAREs and SNARE accessories on the overall fusion process. While this shares weaknesses common to other bulk assays, this technique helped identify the SNARE complex as the necessary core of the fusion machinery (7). More recently, in concert with TIRF microscopy, it has led to the

development of many single vesicle assays capable of monitoring individual steps of SNARE-mediated membrane fusion.

1.5.1 Vesicle-to-suspended bilayer lipid mixing assay

Many single vesicle TIRF assays utilize a vesicle-to-vesicle strategy, similar to that in the bulk lipid mixing assay. In this, t-vesicles are immobilized on an imaging surface, and v-vesicles are injected into a flow cell. A high-speed camera is used to monitor the flow cell as v-vesicles interact with the t-vesicles within (73) (Figure 7).

As hoped, the vesicle-to-vesicle TIRF assay provided many advantages over the old bulk lipid mixing assay. Researchers are able to distinguish between individual docking and lipid mixing events, permitting observation of the impact of SNAREs and SNARE accessories on distinct early stages of fusion (73, 74). However, this technique is not without flaws. Most notably, the high curvature of the target membrane fails to accurately mimic the relatively planar plasma membrane, which may dramatically alter the ability of the two membranes to fuse. Furthermore, αS and other SNARE accessory proteins have membrane affinities highly dependent on membrane curvature, suggesting that their regulatory interactions with the SNARE complex under the conditions imposed by the vesicle-to-vesicle assay differ from those seen *in vivo* (58, 68).

In recent years, planar supported lipid bilayers have been developed as a physiologically relevant alternative more accurately representing the neuronal plasma membrane. Early methods involved formation of an unlabeled bilayer with reconstituted t-SNAREs directly on a solid surface. V-vesicles with fluorescently labeled lipids could then be allowed to flow across the

surface, with docking events observed through their immobilization and lipid mixing observed through two-dimensional dispersal of the dye through the supported bilayer (75, 76).

Unfortunately, the early vesicle-to-supported-bilayer assay had a number of flaws, reducing its usefulness. By directly reconstituting the bilayer on the surface of the solid medium, the mobility of the membrane and membrane-bound SNAREs and SNARE accessory proteins were severely limited. The bilayers also tended to form with high asymmetry caused by irregularities in the solid surface, which further perturbed and interfered with the behavior of lipids and proteins (77). This ultimately prevented accurate recreation of many aspects of SNARE biochemistry, and the assay saw limited use.

However, later developments addressed several of the issues inherent to the supported bilayer assay. In particular, the addition of PEG lipids used to suspend the membrane above the surface permitted increased mobility, increased bilayer symmetry, and limited surface interference. The suspended lipid bilayer thus results in a more physiologically relevant local environment capable of reproducing SNARE-mediated fusion on millisecond timescales (62, 76, 77) (Figure 8).

With recent refinements of the suspended bilayer assay, it has been used to great effect in examining the roles of accessory proteins in membrane fusion. It was this technique, for example, that assisted in the identification of non-aggregated αS in high concentrations as a docking promoter (13, 54). However, this assay does still have its limitations. As lipid merger can occur without opening of the fusion pore, this assay is incapable of investigating fusion pore dynamics, and it can not necessarily determine whether full fusion of the vesicle to the bilayer is occurring (34, 78, 79).

1.5.2 Vesicle-to-suspended bilayer content mixing assay

In order to perform single molecule studies capable of detecting the opening of the fusion pore, it is necessary to develop a method to avoid the limitations of lipid mixing. In vesicle-to-vesicle experiments, this solution came in the form of the content mixing assay (Figure 9). Rather than using FRET-paired lipid dyes to label the vesicle membranes and using an increase in intensity to detect membrane merger, v-vesicles are instead reconstituted with a solution containing sulforhodamine B (SRB) content dye at a high concentration, sufficient to cause self-quenching of the dye. After injection of vesicles into the flow cell, v-vesicles then dock and fuse with unlabeled t-vesicles, releasing the SRB into the t-vesicle, reducing the concentration. This causes de-quenching to occur, permitting detection of the fusion pore opening through a measurable increase in intensity (79, 80).

While useful for demonstrating the ability of these events to proceed beyond initial lipid merger, the technique provides a very limited view of fusion pore dynamics and fails to paint a clear picture of how the pore evolves beyond the initial opening. Furthermore, it suffers from all the limitations of the vesicle-to-vesicle lipid mixing assay in that the curvature of the t-vesicle membrane fails to accurately represent the physiological system (76). As an alternative, some have proposed a modification of the vesicle-to-suspended-bilayer assay. Taking cues from the vesicle-to-vesicle content release assay, it would discard the lipid dye and instead utilize SRB as a content dye, possibly conjugated to dextran chains in order to limit the speed of diffusion and increase the pore size necessary for release of the dye. The release of SRB could then be detected with the opening and expansion of the fusion pore, resulting in an intensity decrease once the dye diffused away from the imaging area. Diffusion of the dye from the vesicle could thus be used to determine size of the fusion pore and how it varies over time.

The suspended bilayer assay has previously been insufficiently robust to permit the development and application of a useful content release variant. However, with recent steps taken to refine the method for analysis of accessory proteins, it appears increasingly promising. With careful calibration, it may be possible to demonstrate a method which can be utilized for research into the fusion pore.

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1.7 Figures

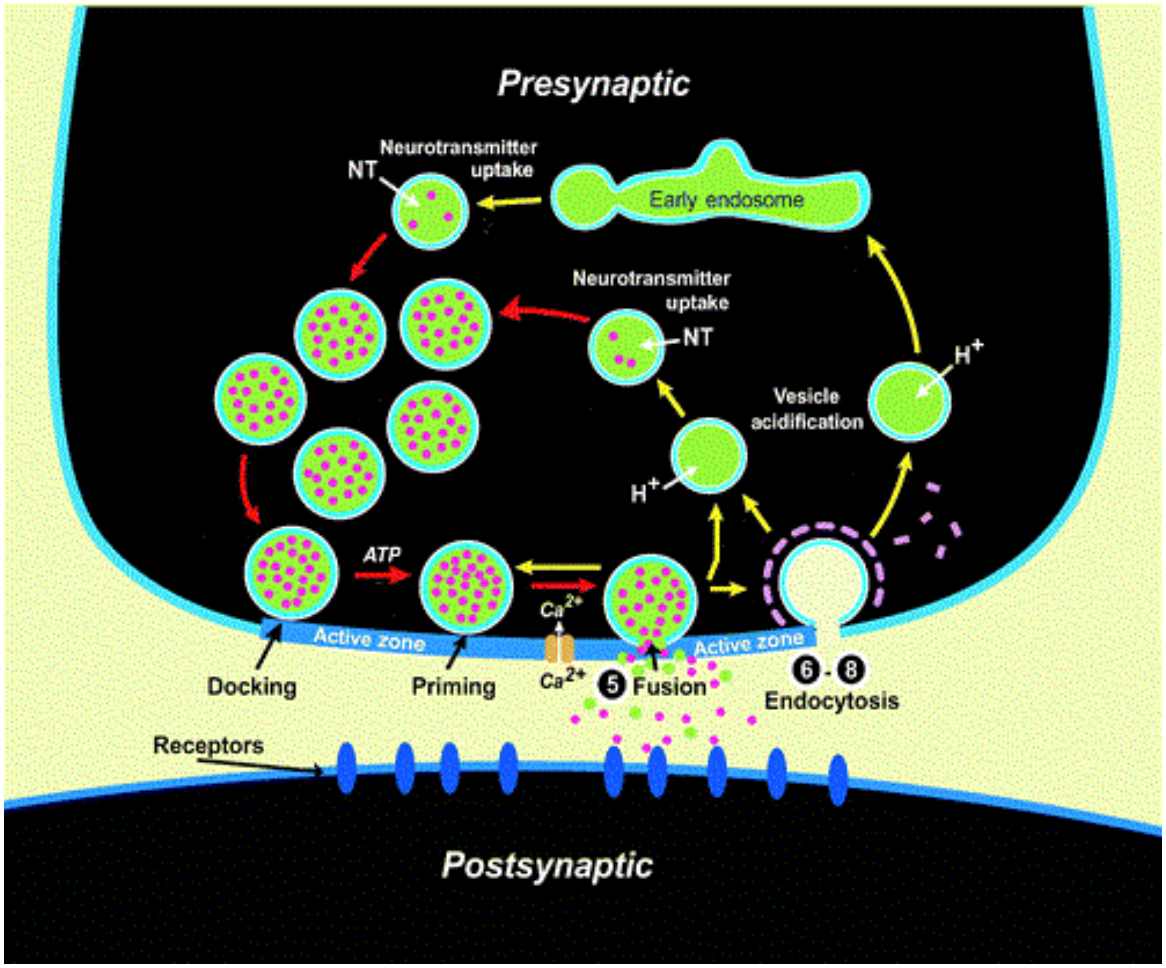

Figure 1. Overview of the synaptic vesicle cycle. Vesicles are filled with neurotransmitters and move to the active zone, where they dock with the pre-synaptic membrane. The vesicle is primed for fusion, which is triggered by the influx of calcium caused by the action potential, releasing neurotransmitters into the synapse where they can be picked up by receptors on the post-synaptic membrane. The vesicle is then recycled according to one of the possible mechanisms and refilled with neurotransmitters for further use. Image modified from (5).

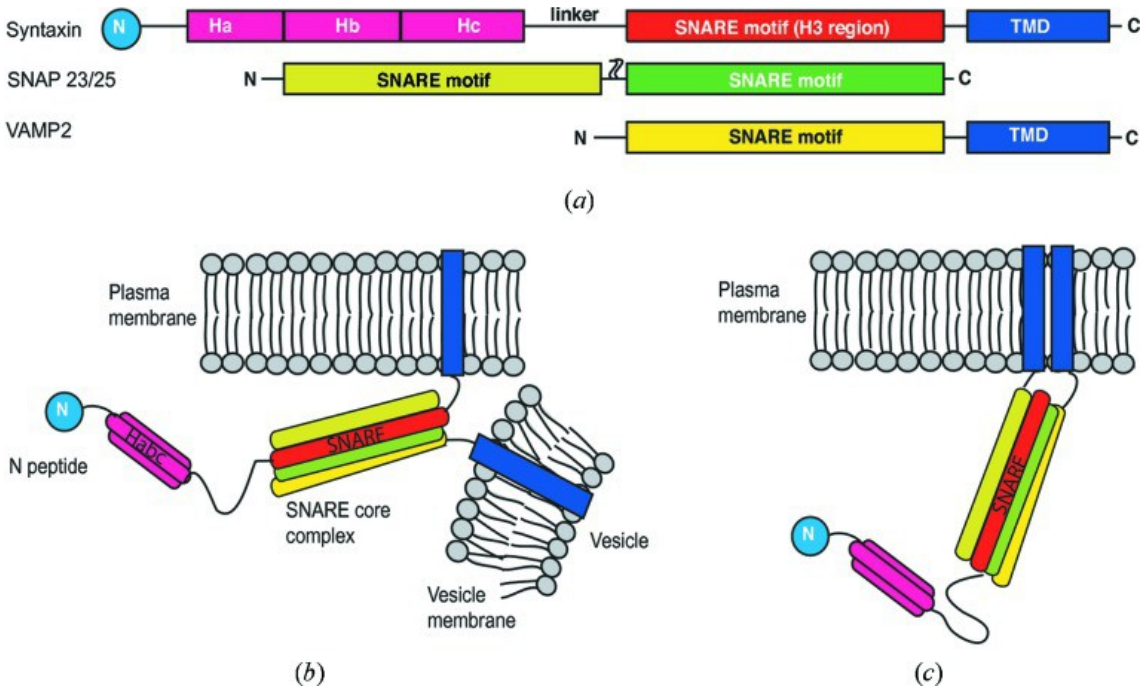

Figure 2. The SNARE complex. (*a*) SNARE domain structures. All SNAREs share the conserved SNARE motif. Transmembrane domains in VAMP2 and Syntaxin are shown in blue. Syntaxin H_{abc} and N-terminal peptide domains are shown in magenta and cyan, respectively. (*b*) Ternary *trans*-SNARE complex. The v-SNARE VAMP2 on the vesicle membrane interacts with the t-SNAREs Syntaxin and SNAP25 on the target membrane to form the four-helix bundle. (*c*) The *cis*-SNARE complex, located on the same membrane after vesicle fusion (81).

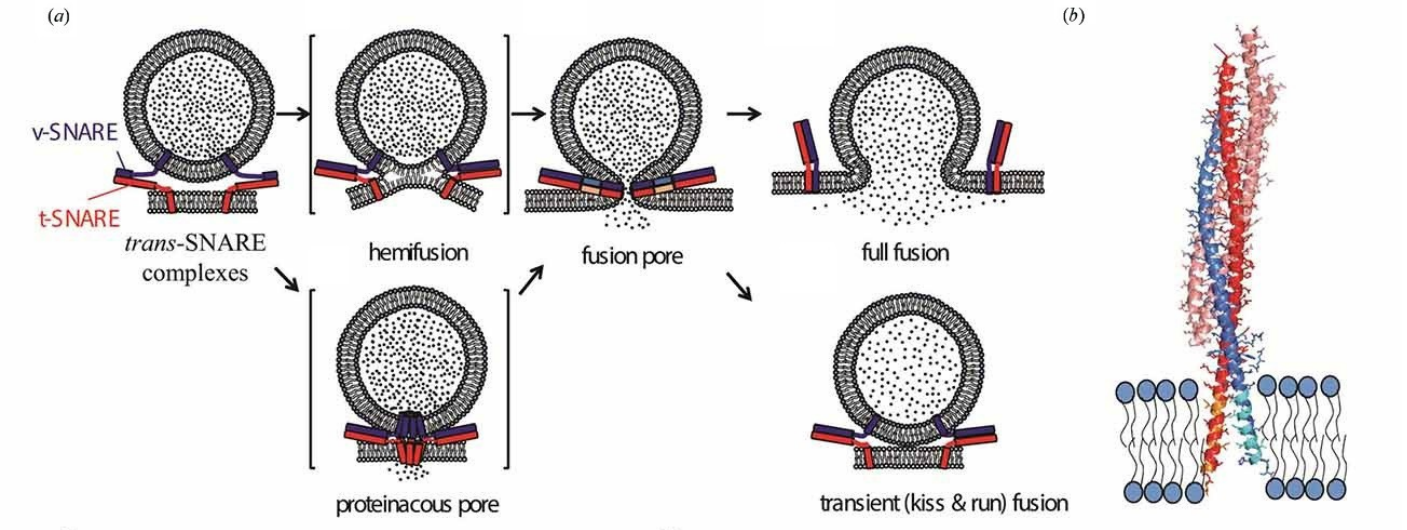

Figure 3. Possible development of the SNARE-mediated fusion pore and structure of the *cis*-SNARE complex. (*a*) Following vesicle docking, SNARE zippering and the action of accessory proteins drive the formation of a fusion pore. The vesicle passes through an intermediate hemifusion stage characterized by lipid mixing, with some proposing a transmembrane domain-lined pore as a hypothetical alternative. After the pore is formed and begins to release content, it may expand further until the vesicle is fully merged to the synaptic membrane in the full fusion model, or close and retreat from the membrane in the transient kiss and run model. (*b*) Crystal structure of the post-fusion *cis*-SNARE complex. STX1A is shown in red, SNAP25 in pink, and VAMP2 in blue. Image modified from (82).

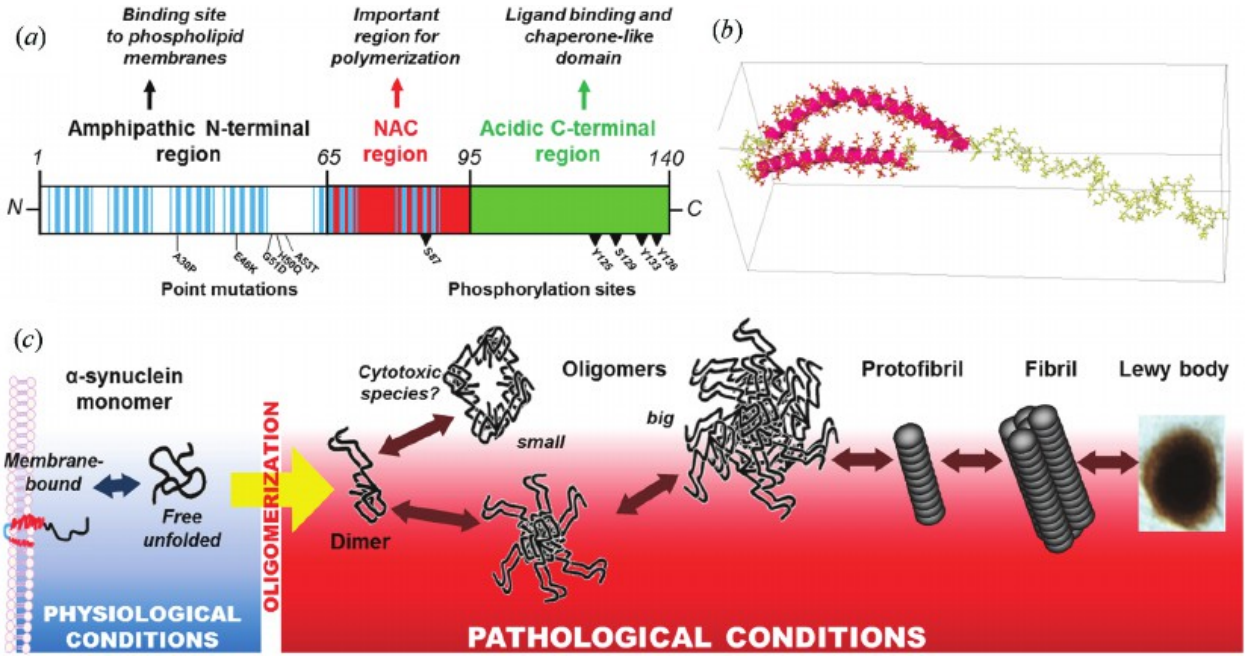

Figure 4. Alpha-synuclein. (*a*) Domain structure of αS. The amphipathic N-terminal domain has a high affinity for lipid membranes, while the C-terminal domain binds to the v-SNARE VAMP2. (*b*) Three dimensional model of αS. The N-terminal domain forms a pair of α-helices, shown in pink, which bind to lipid membranes (83). (*c*) Under pathological conditions, conformers of αS oligomerize into large plaques and Lewy bodies characteristic of several neurodegenerative diseases. Image modified from (84).

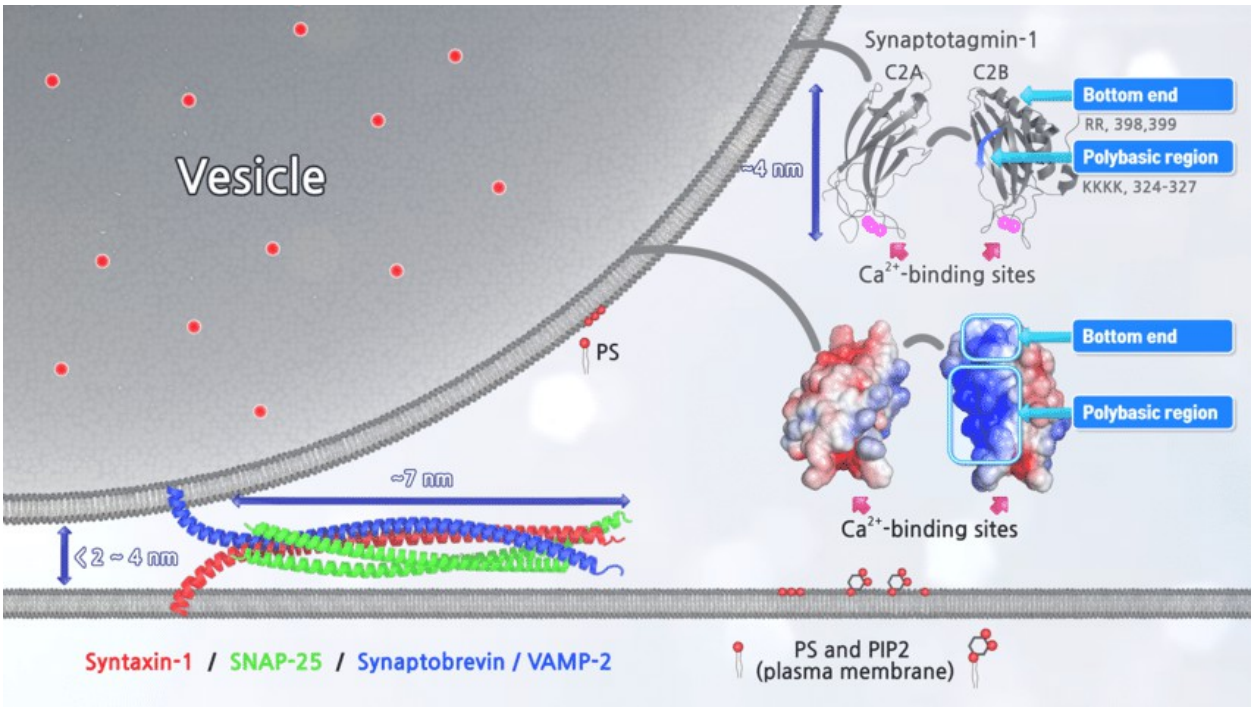

Figure 5. Synaptotagmin 1 on the synaptic vesicle. Syt1 is attached to the vesicle membrane by a trans-membrane domain, shown in grey. Ca^{2+}, shown in pink, attaches to one of the binding sites on the two C2 domains, which are themselves connected by a small linker region. Up to five Ca^{2+} ions may bind a single Syt1, with three binding to the C2A domain and two binding to the C2B domain. The polybasic region of Syt1, indicated in blue, is believed to be responsible for interactions with t-SNAREs (85).

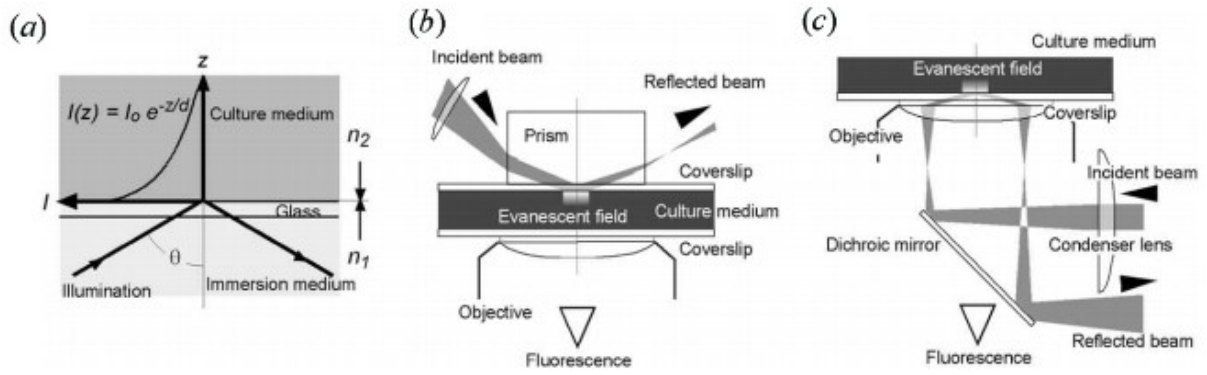

Figure 6. Overview of total internal reflection fluorescence (TIRF) microscopy. (*a*) An evanescent field is generated by total internal reflection as the angle of incident light increases beyond the critical angle. The intensity of the field (I) then decays exponentially with the distance (z) from the intersection of the two media, with a decay length (d) dependent on the incident angle (θ). The exponential reduction in intensity reduces background noise away from the intersecting surface. (*b*) Prism-type TIRF configuration. (*c*) Objective-type TIRF configuration. Image modified from (86).

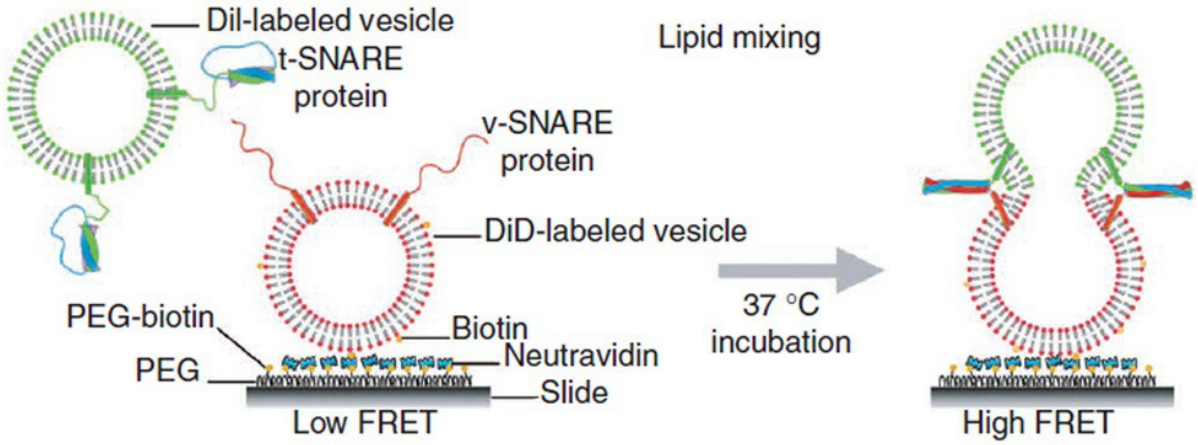

Figure 7. Single vesicle-vesicle lipid mixing assay. DiI (Donor)-labeled vesicles are injected into a flow channel containing DiD (acceptor)-labeled vesicles immobilized on the surface of a slide. SNAREs reconstituted on the vesicles are allowed to interact, and fusion is observed through an increase in FRET. Image modified from (73).

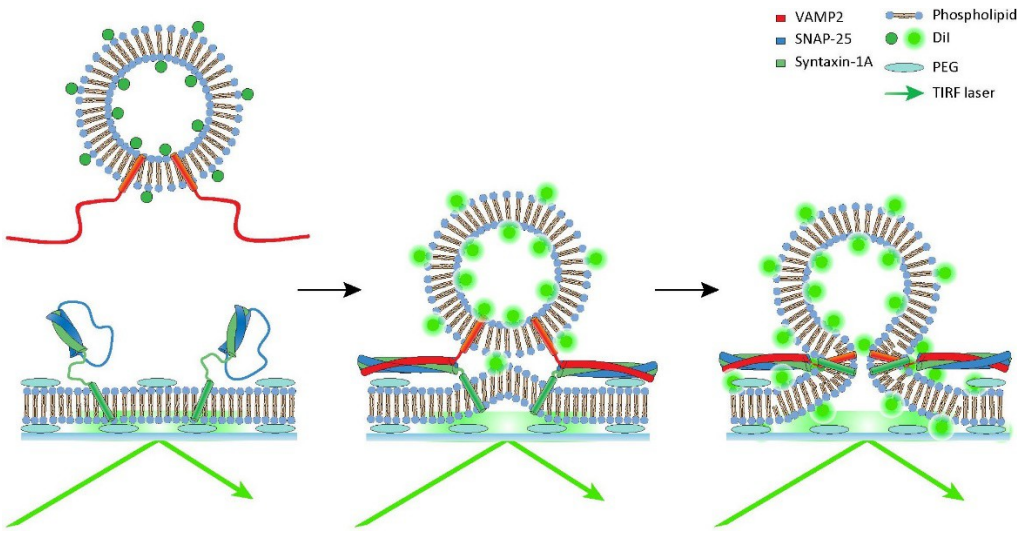

Figure 8. Single vesicle-to-supported bilayer lipid mixing assay. DiI-labeled v-vesicles are injected into a flow channel containing a PEG-suspended t-bilayer. The TIRF laser excites the dye, and lipid merger can be detected as the labeled lipid disperses in the bilayer. Image modified from (54).

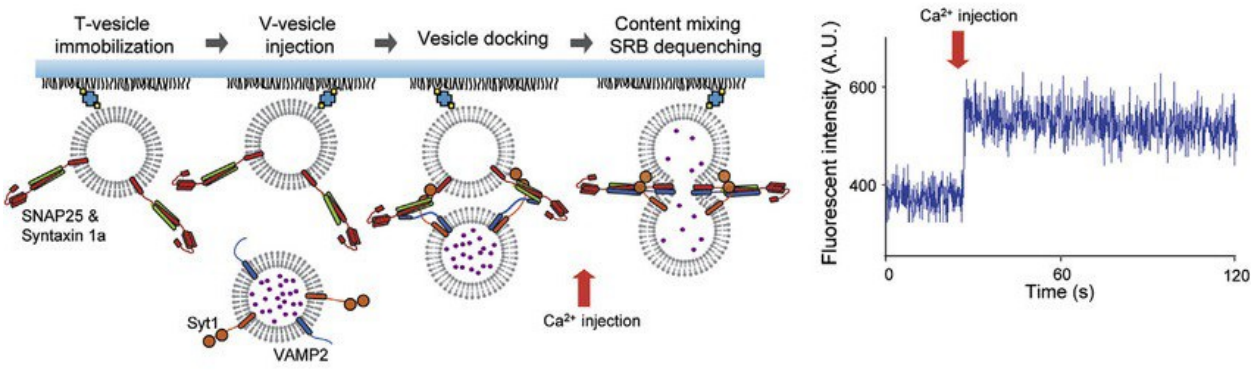

Figure 9. Single vesicle-vesicle content release assay. V-vesicles containing SRB at a concentration sufficient to induce self-quenching are injected into a flow channel containing immobilized t-vesicles. Fusion can be detected using the diffusion of SRB from the v-vesicle to the t-vesicle through the fusion pore. The reduction in SRB concentration results in de-quenching of the dye and a measurable increase in fluorescent intensity. Image modified from (87).

CHAPTER 2

NOVEL SINGLE VESICLE TO SUSPENDED BILAYER CONTENT RELEASE ASSAY PROBES FUSION PORE DYNAMICS

2.1 Abstract

Neuronal SNARE-mediated vesicle fusion involves the highly regulated formation of a fusion pore permitting the release of neurotransmitters into the synapse. Dilation of this fusion pore is believed to be one of the most energy intensive steps in the entire process. However, the mechanisms regulating formation and expansion of the fusion pore are poorly understood, and work on the topic has been limited by the scarcity of available methods. Here, we present a new single vesicle *in vitro* fusion assay capable of studying SNARE-mediated fusion pore dynamics. Unlike previous vesicle-to-vesicle content release assays, we demonstrate that our suspended bilayer assay can be used to observe both expansion and collapse of the fusion pore after its initial formation, without interfering membrane curvature effects. We further show that our assay is capable of probing the roles of accessory proteins, with a demonstrable increase in the net time of the fusion pore in its dilated state using alpha-synuclein (αS). Curiously, the assay suggests neither the SNARE-only nor the SNARE-plus-αS system are capable of regularly driving the vesicle towards full, fast content release, indicating other accessory proteins may be necessary, though both systems show fusion pore fluctuations which may be exploited. We thus demonstrate that the vesicle-to-suspended-bilayer content release assay is a promising platform for further studies of the fusion pore, and will prove useful in elucidating the roles of additional accessory proteins in modifying pore dynamics.

2.2 Introduction

The function of the central nervous system depends upon neurotransmission. Sophisticated networks of neurons communicate through the highly regulated fusion of lipid vesicles to the pre-synaptic plasma membrane, releasing neurotransmitters into the synapse through a fusion pore where they can be picked up by receptors on the post-synaptic membrane. This serves to propagate signals between neurons, coordinating physical activity and giving rise to cognitive thought. However, many aspects of the systems mediating neurotransmission are poorly understood, amongst them the dynamics and regulation of the fusion pore itself (1, 2, 3). This is particularly troublesome, as defects in neurotransmission regulation are known to be a primary factor in many serious neurological diseases (4, 5). A better understanding of the mechanisms involved will lead to greater knowledge of the underlying causes of these diseases and suggest new potential avenues for treatment.

The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) complex is believed to be the minimum machinery necessary for synaptic vesicle fusion and neurotransmitter release (6, 7). Vesicle associated (v-) SNAREs on the neurotransmitter-containing vesicle bind to target (t-) SNAREs on the surface of the pre-synaptic plasma membrane, forming a helical coiled coil. The energy released by the spontaneous formation of the coiled coil is believed to drive lipid merger and the formation of the fusion pore (8, 9, 10). This pore is believed to develop according to one of two proposed mechanisms. The first is a 'kiss-and-run' mechanism whereby the vesicle forms a small pore ~1-2 nm in diameter, releases content, then seals the pore and eventually disengages from the plasma membrane (11, 12, 13). The second is a 'full fusion' or 'exocytosis/endocytosis' mechanism in which, after formation of

the fusion pore, the pore continues to dilate until the vesicle has been fully incorporated into the pre-synaptic plasma membrane and all content has been released into the synapse. In either case, the vesicles are later retrieved and recycled for further release events, though the mechanism by which this occurs differs (12, 14, 15). Current theory suggests that fusion pore dilation is the single most energy demanding step in synaptic vesicle fusion, raising questions as to whether SNAREs are sufficient to drive the process beyond the initial pore opening (16), but a lack of biochemical assays capable of probing fusion pore dynamics has severely limited investigation of the process. Thus, the extent to which either mechanism predominates in neurotransmission under different circumstances remains unclear, and the roles SNARE auxiliary proteins play in regulating fusion pore dynamics and promoting one exocytosis mechanism over the other are poorly explored (11, 17, 18).

Alpha-synuclein (αS) is one such protein relevant to studies of SNARE-mediated fusion. A soluble, intrinsically disordered protein, αS is best known for pathologically aggregating into neuronal plaques and Lewy bodies, and as such has been of major interest in studies of Parkinson's and other neurodegenerative diseases characterized by similar aggregates (19, 20). However, its normal function has not been firmly established, despite its large non-aggregated presence in healthy neurons and heavy localization at the pre-synaptic terminal. Past studies suggest it plays a role in vesicle recycling, and recent work indicates that non-aggregated αS also promotes docking by cross-bridging the vesicle and target membrane (21-24). Given its known affinity for membrane curvature and ability to deform membranes, some have proposed that αS plays a role in fusion pore dilation. However, recent studies of the topic have been limited in scope (24, 25), and more needs to be done to explore the role of αS in the dynamics of the fusion pore after docking occurs.

Much of the past single vesicle work exploring opening of the fusion pore utilized a vesicle-to-vesicle content release assay. In this setup, vesicles reconstituted with t-SNAREs are immobilized on a PEG-coated surface. Non-immobilized vesicles containing self-quenching fluorescent content dye and reconstituted with v-SNAREs are then allowed to flow over the surface, where they can interact with the immobilized vesicles. Fusion between two vesicles could be detected upon opening of the fusion pore and diffusion of the content dye into an immobilized vesicle, causing de-quenching of the content dye as its concentration dropped with a corresponding increase in intensity (26, 27). This was used to good effect in studies of the Ca^{2+}-sensor Synaptotagmin 1 (Syt1) and its role in Ca^{2+}-triggered fusion, confirming that pore opening occurred and indicating that Syt1 served to promote SNARE-mediated pore opening in the presence of Ca^{2+} (28, 29). However, the vesicle-to-vesicle assay could not readily distinguish the dynamics of the large pore following the initial opening, a weakness also shared with the patch clamp electrophysiology technique used in studies of small fusion pore opening (30). In addition, the high curvature of the target membrane fails to accurately represent the relatively planar plasma membrane, altering fusion dynamics and impacting the ability of accessory proteins to perform their physiological function (31).

Attempts to rectify the latter problem using a suspended t-bilayer coating a solid imaging surface instead of immobilized t-vesicles have proven extremely promising. However, a lack of refinement has so far limited its application primarily to lipid mixing assays (32, 33). While these are capable of studying docking, hemifusion, and membrane lipid exchange, and while the last of these has often been used as a proxy for subsequent opening of the fusion pore, lipid mixing can actually occur without any pore formation whatsoever (34). As such, the current assays are insufficient to probe fusion pore dynamics, and progress in this area has been limited.

In order to study fusion pore dynamics, we developed a novel single-vesicle-to-suspended-bilayer content release assay capable of overcoming the limitations on past *in vitro* content release studies. Unlike the vesicle-to-vesicle system, the suspended bilayer provides a physiologically relevant environment for the study of SNARE-mediated membrane fusion through visualization of individual vesicles on a millisecond timescale. In addition, unlike the vesicle-to-vesicle or the suspended bilayer lipid mixing systems, the suspended bilayer content release assay permits resolution of individual steps in pore formation and dilation through the release and diffusion of the fluorescent content dye from docked vesicles.

Using this assay, we determined that the net duration of pore dilation and number of dilation events per docked vesicle increased in the presence of αS when compared to SNAREs alone, with a corresponding increase in the net loss of content dye. Furthermore, we found that many vesicles failed to release SRB-10 kDa dextran after docking in the absence of αS, suggesting that SNAREs alone are insufficient to regularly drive dilation of the fusion pore. Neither system saw regular full release of dye except over large (>1 s) timescales. Taken in context, it is likely that other accessory proteins are necessary to drive fusion pore dilation, and the vesicle-to-suspended-bilayer content release assay provides a viable platform to study them.

2.3 Methods and Materials

2.3.1 Plasma Constructs and Site-Directed Mutagenesis

DNA sequences encoding syntaxin 1A (amino acids 1-288 with three native cysteines replaced by alanines), SNAP-25 (amino acids 1-206 with four native cysteines replaced by alanines), SNAP-25E (amino acids 1-180), VAMP2 (amino acids 1-116 with one native cysteine replaced by alanine), soluble VAMP2 (amino acids 1-96), and αS (amino acids 1-140) were

inserted into a pGEX-KG vector as N-terminal glutathione S-transferase (GST) fusion proteins. A DNA sequence encoding Syt1 (amino acids 50-421 with four native cysteines replaced by alanines and one native cysteine replaced by serine) was inserted into a pET-28b vector as a C-terminal polyhistidine (His)-tagged protein. DNA sequences were confirmed by the Iowa State University DNA sequencing facility.

2.3.2 Protein Purification

All N-terminal GST recombinant neuronal SNARE proteins and αS were expressed in *Escherichia coli* BL21 (DE3) cells. These cells were grown at 37°C in LB (Luria-Bertani) medium with 100 μg/mL ampicillin until the absorbance at 600 nm reached 0.6–0.8, and induced to express overnight through addition of IPTG (isopropyl β-D-thiogalactopyranoside, 0.3 mM final concentration) at 16°C. Cells were pelleted and resuspended in 15 ml of high salt PBS (HSPBS) (497 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for soluble proteins or 15 ml of high salt PBST (HSPBST) (pH 7.4, HSPBS containing 0.2% Triton X-100) for membrane proteins with final concentrations of 1 mM AEBSF ([4-(2-aminoethyl) benzenesulfonyl fluoride]) and 4 mM DTT (dithiothreitol). Cells were then lysed by homogenization and centrifuged at 25,000 x*g* for 30 minutes at 4°C.

Following lysis and centrifugation, the supernatant was collected and mixed by nutation for 2 h at 4°C with 1 ml of glutathione agarose beads in HSPBS, if purifying a soluble protein, or 1 ml of glutathione agarose beads in HSPBST if purifying a membrane protein. After washing the protein intensely with HSPBS or HSPBST as appropriate, the protein was cleaved overnight by 0.02 unit/μl thrombin at 4°C. The protein was then eluted in PBS if a soluble protein, or PBS containing 0.8% noctyl-D-glucopyranoside (OG) if a membrane protein. Concentration was determined via DC assay.

The αS was concentrated to ~1 ml after this process and loaded onto an AKTA FPLC with a GE Superdex 200 Increase 10/300 column for size-exclusion chromatography exchanging αS into PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Individual fractions were collected and an 15% SDS-PAGE gel was used to identify fractions lacking high molecular weight bands indicative of oligomerization, as previously described (23). These fractions were combined and concentrated.

The C-terminal His-tagged Syt1 was expressed and purified in a manner identical to the N-terminal GST proteins above, save that it used Ni-NTA beads instead of glutathione agarose. The Ni-NTA column was washed with HEPES (25 mM HEPES, 20 mM imidazole, 400 mM KCl, pH 7.4), and Syt1 was eluted with 500 mM imidazole (25 mM HEPES, 400 mM KCl, pH 7.4).

All protein samples had glycerol added to a final concentration of 15% and were stored at -80°C.

2.3.3 Membrane Reconstruction

The t-bilayer was made using a mixture of POPC (1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine), DOPS (1,2-dioleoyl-sn-glycero-3-phosphatidylserine), PIP_2 (phosphatidylinositol 4,5-bisphosphate), and PEG2000 (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) in chloroform at a molar ratio of 78:15:2:5. The v-liposome was made using a mixture of POPC, DOPS, and cholesterol in chloroform at a molar ratio of 54:5:40. Both lipid mixtures were first dried under an air stream, then dried in a vacuum overnight. The t-bilayer lipid was resuspended in HEPES (25 mM HEPES/KOH, 150 mM KCl, pH 7.4) + 1% OG (Octyl-beta-glucoside). The v-liposome lipid

was resuspended in HEPES with 10 kDa sulforhodamine B-dextran (SRB-dextran, 30 μM) before undergoing 10 flash freeze-thaw cycles, moving between liquid nitrogen and boiling water. Protein-free large unilamellar vesicles (~100 nm diameter) were prepared by extrusion through polycarbonate filters to make the v-liposomes.

For the t-bilayer, syntaxin-1A and SNAP-25 were premixed in a molar ratio of 1:1.5, and the mixture was left at room temperature for 30 minutes to form the t-binary complex prior to reconstitution. The t-bilayer lipid was added to the t-binary complex at a lipid-to-complex ratio of 2000:1. The mixture was then diluted via addition of three times the volume of the mixture in HEPES. The mixture was then dialyzed overnight at 4°C in 2L of HEPES containing Bio-Beads™ SM-2 Resin.

For functional v-vesicles, v-liposomes were mixed with VAMP2 or 1:1 VAMP2:Syt1 at a lipid-to-protein ratio of 200:1. The mixture was then diluted via addition of three times the volume of the mixture in HEPES with 30 μM SRB-dextran. This mixture was then dialyzed overnight as above.

2.3.4 Vesicle-to-Suspended Bilayer Content Release TIRF Assay

A quartz slide and glass cover slip were cleaned and hydroxylated by boiling in piranha solution (1:1 mixture of concentrated sulfuric acid and 30% hydrogen peroxide) for 15 minutes. Slides and cover slips were rinsed with ddH_2O and placed in a sonicator for 30 minutes to remove residual acid. After another rinse with ddH_2O, slides were dried and prepared with double-sided tape and dried cover slips to generate microfluidic flow chambers. Chambers were then filled with t-bilayer mixture prepared from the overnight dialysis and left at room temperature for 2 h to allow formation of the bilayer. Excess mixture was gently washed out of

the flow chambers at a rate of 50 µl/min using HEPES or HEPES with 5 µM αS as appropriate. Washed slides were incubated while heating at 37°C for 2 h.

The quartz slide was then placed on the imaging stand of our total internal reflection fluorescence (TIRF) microscope, which regulated the temperature of the slide to 37°C. Imaging oil was put on the microscope prism, and the prism was lowered onto the quartz slide. Following adjustment of the incident angle and intensity of the 532 nm exciting laser, real-time image acquisition was initiated with an imaging area of ~110 x ~110 µm and a time resolution of 20 ms. Post-dialysis v-vesicles, diluted to an experimental concentration in HEPES or HEPES with 5 µM αS, were injected into the flow chamber at a rate of 50 µl/min, stopping the pump promptly following first observation of events to prevent vesicle merger from the flow effect. Images were collected for 60 to 180 s in 60 s movies and analyzed for docking and content release events using custom-built analysis software.

2.3.5 Data Analysis

The fluorescence of SRB from the v-vesicles was monitored to determine docking and content release events using in-house MATLAB® 2014 (b) and 2019 (a) analysis software. Each recording was analyzed frame by frame based on both visual determination and fluorescence trace pattern analysis. Changes in fluorescence intensity were used to determine docking, fusion pore dilation, and fusion pore collapse. Both visual and trace analysis had to indicate an event for it to be counted. Faux-events less than twenty intensity units above baseline with a simple intensity plateau trace prior to dropping immediately back to baseline were discounted from the analysis.

Docking events are indicated by a vesicle immobilized on the bilayer, with a matching increase in measured fluorescence at the docking location. Release events were defined as the diffusion of dye away from the vesicle, coupled with an increase in intensity relative to the amount of dye being released and subsequent drop in intensity as this amount decreased until the release event ended. Increases and decreases in dye release under this system were used to distinguish between pore dilation and contraction during a release event, respectively. Docking-only events and post-release event vesicles faded over time due to photo-bleaching or non-visible release of linearized SRB-dextran, or experienced rapid, single frame declines to baseline intensity and visual disappearance without content release defined as SNARE complexes disassembling and the vesicle retreating from the bilayer.

The in-house software was used to extract data related to the number, duration, and intensity of all measured events after analysis. Data was plotted into histograms and used to compare pore dynamics between SNARE-only and SNARE-plus-αS systems.

2.4 Results

2.4.1 A Novel Single Vesicle-to-Suspended Bilayer Content Release Assay

Many previous *in vitro* attempts to study the SNARE-mediated vesicle fusion pore utilized a vesicle-to-vesicle content release assay reliant on fluorescent dye dequenching as an indicator for pore opening. While this method helped demonstrate that the role of synaptotagmin in Ca^{2+}-triggered exocytosis extended to the fusion pore (29), and while it saw some attempts to apply it to other accessory proteins, it still held significant drawbacks. The experimental setup and high membrane curvature limited the physiological relevance of the studies and failed to demonstrate an accurate view of pore dynamics. While alternatives existed, they were similarly

relegated to the study of fusion pore opening, and of limited use in probing the following evolution of the pore (30, 33, 35).

In order to resolve these issues, we developed a system utilizing a planar bilayer for the target membrane, similar to past vesicle-to-suspended bilayer assays used in membrane merger experiments and capable of more accurately mimicking the geometry of the physiological plasma membrane. In this assay, a planar bilayer reconstituted with the t-SNAREs SNAP-25 and syntaxin-1A was prepared in a flow chamber on a quartz slide. A polyethylene glycol (PEG) cushion, formed by PEGylated lipids in the bilayer, served to suspend this t-bilayer above the slide in order to permit reconstitution of the t-SNAREs in their proper positions and maintain the desired membrane fluidity (36). After the bilayer was formed, vesicles reconstituted with VAMP2 were slowly injected into the flow cell to avoid disturbance of the bilayer, and the fusion of these v-vesicles to the t-bilayer was observed using total internal reflection fluorescence (TIRF) microscopy.

After injection of the v-vesicles into the flow chamber, images of the slide were taken in 20 ms intervals for 60 s. Individual docking and release events were detected using fluorescent SRB-dextran, initially encapsulated in the vesicles at 30 μM, below the self-quenching concentration used in vesicle-to-vesicle experiments (37, 38). The SRB caused an increase in fluorescence as the vesicle docked to the surface. Content release from opening and expansion of the fusion pore was characterized by visual diffusion of dye and a brief increase in fluorescence, likely due to the exponential decay of the evanescent field causing the dye to appear brighter as it drew closer to the imaging surface (39), followed by a subsequent decrease below the pre-release docked vesicle intensity due to the overall reduction in dye contained in the vesicle. The dextran conjugation limited the speed of SRB diffusion to permit visual

observation, and increased the hydrodynamic radius of the dye in order to permit detection of fusion pore dilation beyond this radius (Figure 1). The intensity, duration, and number of release events from the large fusion pore were recorded for each detected vesicle and used in further analysis.

To ensure that observed fusion was related to the activity of the SNARE complex, control experiments in which the t-bilayer was prepared without syntaxin 1A or SNAP-25, or the v-vesicles were prepared without VAMP 2, were performed. These controls failed to demonstrate content release or true docking events. Observed faux-docking events, present in all control and full-SNARE systems as brief low-intensity plateaus without any content release and which dropped from plateau to baseline in a single frame, were discarded from analysis as noise.

2.4.2 SNAREs Drive Fusion Pore Opening, but Dilation is Limited

As the controls demonstrate, the SNARE complex is necessary for vesicle fusion to occur. Past work has indicated that SNARE zippering is capable of driving lipid merger (6, 9). In order to determine whether SNAREs alone are sufficient for driving fusion pore formation and dilation, we applied our content release assay to the full system with syntaxin 1A, SNAP-25, and VAMP 2 all reconstituted with the appropriate membranes. V-vesicles were injected into the flow chamber and interactions with the t-bilayer were observed through excitation of the fluorescent content dye. These interactions were characterized through visual study and analysis of the associated fluorescent intensity traces (Figure 2).

Using the 10 kDa dextran-conjugated SRB, we observed that around half of the analyzed vesicles failed to demonstrate any measurable release events. Of the remainder, none demonstrated an initial release sufficiently large to expel all content dye from the interior of the

vesicle before the release event ended. These results are particularly notable given the long duration of observed release events, on the order of hundreds of ms to over 1 s, well beyond the typical *in vivo* timescale for neurotransmission (40, 41). This suggests pore size was only just sufficient to permit release of SRB-dextran before the pore contracted and visible release halted.

Another notable observation came in the form of the number of release events per vesicle. While a plurality of those vesicles with observable content release was limited to only a single release, other vesicles demonstrated anywhere between two and nine distinct content release events while remaining docked to the t-bilayer. The number of frames between individual release events differed, as well. While most followed within ~200 ms of the prior release event, others followed over ~1000 ms after the end of the prior release. This finding indicates that, after initial pore opening and dilation, the pore is contracting and dilating multiple times without closure and separation of the vesicle from the bilayer or proceeding to full vesicle fusion in line with past suggestions of pore fluctuation (42, 43).

We also observed a number of rapid, partial drops in intensity within a single frame without disappearance of the vesicle or visible release of dye. As these events lacked obvious release, and intensity drops were sufficiently large that any such release would have been observed, the cause of this decrease was unclear. Disassembly of the SNARE complex and retreat of the vesicle from the bilayer may be involved, and would result in a decrease in measured intensity due to exponential decay of the TIRF evanescent field. While for some vesicles this resulted in a rapid return to baseline intensity within the measured area, others maintained a steady intensity or returned to baseline without rapid drops over the course of hundreds of frames. Both occurred in vesicles that demonstrated at least one visible release event and those that failed to demonstrate any. The extent to which partial linearization of the

dye and non-visible slow release are responsible versus photo-bleaching of the dye for this intensity decay in different vesicles was not determined.

However, this did raise the question as to whether the fusion pore was still opening in the docked vesicles with no visible release events, and the failure to release was instead caused by insufficient pore dilation. Instead of utilizing SRB-dextran, unconjugated SRB was encapsulated in the v-vesicles as a content dye, and the vesicles were injected into the flow cell as described above. If fusion pore opening was occurring, the small hydrodynamic radius of unconjugated SRB would allow it to diffuse from the vesicle in cases where the pore failed to dilate enough to allow release of the large SRB-dextran. However, this small size also prevented direct observation of release due to the high rate of diffusion and camera speed limitations.

No docked vesicles were reliably observed when using SRB content dye. In order to determine whether this was due to a failure of the system or due to release of dye through a small fusion pore, myricetin was allowed to mix with the v-vesicles prior to injection. Myricetin is a small flavonoid commonly derived from plants, capable of binding to the SNARE complex and arresting the vesicle in a hemifusion state, preventing pore opening (44). In our system, the plus-myricetin unconjugated SRB v-vesicles showed docking similar to that found in those utilizing SRB-dextran, demonstrating that the failure to observe docking events using unconjugated SRB in the absence of myricetin was due to rapid content release. This further suggests that a fusion pore formed in all or almost all detected docked vesicles containing SRB-dextran, with only a fraction of the population experiencing pore dilation sufficient for release of the dye.

2.4.3 Rapid Ca^{2+} Triggered Full Content Release with Synaptotagmin 1

The membrane protein Synaptotagmin 1 (Syt1) is a Ca^{2+} sensor located on vesicle membranes, and is known to participate in SNARE-mediated vesicle fusion. Previous work has indicated that, in the presence of Ca^{2+}, Syt1 promotes instantaneous fusion and fusion pore dilation (29, 45, 46). In order to corroborate these results, demonstrate that our system is capable of showing full content release from a larger fusion pore, and further determine whether SNAREs are sufficient for driving pore dilation, we reconstituted Syt1 into v-vesicles alongside VAMP 2 and injected them into the flow cell with 500 μM Ca^{2+},

A subset of analyzed vesicles under these conditions failed to release dye or displayed patterns of multiple small releases, similar to those found in the absence of Syt1. However, unlike with SNAREs alone, a population of vesicles demonstrated full release of content dye in a single large release event (Figure 3). All internal content dye diffused away from the analyzed location in less than ten frames (~200 ms) in many of these, indicating pores dilated to a size not observed in the SNARE-only system.

2.4.4 αS Promotes Release by Stabilizing Vesicle on Bilayer

Alpha-synuclein has previously been shown to enhance vesicle docking and SNARE-complex formation (22, 23), but an understanding of how it interacts with fusion pore dynamics is limited. The ability of αS to distort bilayers might suggest it plays a key role in fusion pore dilation (25, 47). As a preliminary exploration, v-vesicles containing SRB-dextran were injected into flow cells containing the t-bilayer in the presence of 5 μM αS and subjected to imaging as described above. The αS was purified by FPLC to remove higher-order oligomers shown to interfere with SNARE-mediated fusion (23).

While the addition of αS did result in somewhat higher initial content releases than those observed with the SNARE-only system, no single full content release events comparable to those seen with Ca^{2+} and Syt1 were observed. However, the number and overall duration of all release events from a single docked vesicle increased notably, with multiple releases from a single vesicle common and almost no analyzed vesicles observed without any release at all. On average, the plus-αS system demonstrated around one more release than the SNARE-only system if non-releasing vesicles were discounted, and two more if non-releasing vesicles were included. Related to this, the overall time of pore opening and the decrease in measured intensity from all observed releases in the presence of αS was much greater than with SNAREs alone (Figure 4).

Despite the initial concentration of SRB-dextran remaining the same across all experiments, the max measured intensity for docked vesicles was much greater with αS than without, with measured averages of ~80 intensity units for the SNARE-only system and ~170 units for the SNAREs-plus-αS. The exponential decay of the evanescent field has a large impact on measured intensity for a given event (36), indicating that the vesicles may have been held closer to the suspended bilayer in the presence of αS, in line with prior evidence suggesting that αS assists in docking (23). Increased bilayer proximity and stabilization on the bilayer could then account for the increased number of individual observed release events, as the fusion pore could reversibly dilate and contract more easily without the vesicle withdrawing from the bilayer.

2.5 Discussion

A comprehensive understanding of fusion pore dynamics has long eluded researchers. While in the past some have suggested that formation of the SNARE complex provides all energy necessary to drive full fusion, the high energy barriers that would be necessary to

overcome for both pore opening and especially pore expansion raised serious questions as to whether this was indeed the case (16). Early single molecule assays have been limited in their ability to elucidate steps beyond hemifusion or the initial opening of the fusion pore, leaving the answer to this question unresolved. However, the development of our modern single vesicle-to-suspended bilayer content release assay provided an excellent opportunity to explore fusion pore dynamics beyond opening, allowing this question to finally be resolved.

By reconstituting VAMP2 into liposomes encapsulating fluorescent 10 kDa SRB-dextran content dye and analyzing individual interactions between these vesicles and a suspended t-bilayer reconstituted SNAP-25 and STX1A, we were able to observe the release of content from the vesicles, suggesting that the SNAREs were indeed capable of driving the opening of a fusion pore without the influence of accessory proteins (6, 17). However, while a variant of the assay using unconjugated SRB suggested that most vesicles saw opening of the pore to some extent, around half of the vesicles containing SRB-dextran failed to visibly release any content at all. Of the rest, none demonstrated the rapid or full release that would be characteristic of continued pore dilation to or even near the point of full vesicle fusion. This suggests that, while SNAREs are sufficient to regularly drive pore opening, they are not sufficient to drive pore expansion far on their own. While they might be capable of some release akin to the kiss-and-run fusion mechanism, full vesicle fusion appears to be unlikely in the absence of the appropriate accessory proteins (12). This raises interesting questions regarding differences in the size of neurotransmitters carried by vesicles, with small molecule neurotransmitters more readily capable of escaping through a small pore driven solely by SNAREs than neuropeptides (48), which may suggest differences in accessory protein concentration or localization depending on vesicle content.

Preliminary work incorporating Syt1 into the v-vesicle appears to support findings with the SNARE-only experiments. Unlike the SNARE-only system, SNAREs-plus-Syt1 demonstrates rapid, full, Ca^{2+}-triggered release of content from v-vesicles as they fuse to the t-bilayer. Past work has indicated that Syt1 promotes fusion in the presence of Ca^{2+}, and this result appears to support that claim (29, 45, 46). However, this could not occur without regular fusion pore expansion beyond that observed with SNAREs alone, suggesting that Syt1 also operated by promoting fusion pore dilation, particularly given that small pore opening without dilation sufficient for release appeared to be common in the absence of Syt1.

However, further analysis of the SNARE-only system demonstrates that the size of the SNARE-mediated fusion pore does not necessarily remain static after formation. Nor does it necessarily always solely contact and close after dilating to its max size. Of those vesicles with observed release events, many demonstrated patterns of multiple distinct releases after the first. This strongly suggests that the size of the fusion pore was in flux, expanding enough to permit the release of some content, then contracting below the hydrodynamic radius of the dye to prevent escape, then expanding and releasing more before contracting once again.

This tendency appears greater in the presence of αS, with an increase in the number of distinct release events per vesicle. Furthermore, unlike the SNARE-only system, almost all docked vesicles in the SNARE-plus-αS system demonstrated at least one visible release event. While past work has suggested that the membrane-deforming properties of αS might play a role in promoting pore dilation (25), caution must be taken in interpreting these results, particularly in light of other collected data. Like the SNARE-only system, but unlike the SNAREs-plus-Syt1, the plus-αS system failed to demonstrate any single full release events, indicating collapse of the fusion pore below the hydrodynamic radius of the dye. Further, while the duration of individual

releases may be somewhat greater with αS, the net increase in release time per vesicle is likely more related to the number of individual releases. This suggests that the influence of αS on pore dilation may be largely indirect, though it does raise the question of how.

A possible suggestion might be taken from examination of the maximum intensities of vesicles in the SNARE-only and plus-αS experiments. Those docking in the presence of αS demonstrated notably higher max intensities, despite the initial internal dye concentration remaining the same across all experiments. This difference in observed intensity was likely due to the exponential decay of the evanescent field used for exciting the fluorescent dye in TIRF. If the vesicles were held more closely to the membrane in the presence of αS, then they would appear brighter than more loosely and distantly held vesicles in the SNARE-only experiments. Previous work has shown that αS does indeed assist in docking, making this quite likely (23). If so, then the role of αS in promoting pore dilation may be closely related to reversible changes in fusion pore size. By helping tether the vesicle to the target membrane, it provides more opportunities for the pore to expand and contract without it closing and retreating enough from the bilayer to prevent further fluctuations (Figure 5).

While uncertain at this stage, other accessory proteins may be able to exploit the fluctuating fusion pore by preventing contraction, allowing the vesicle to proceed to full fusion without shifting back to closure. Though still in its early stages, the single vesicle-to-suspended bilayer content release assay has been shown to be capable of exploring the roles these accessory proteins have in SNARE-mediated fusion pore dynamics. Further explorations utilizing this new assay could greatly enhance our understanding of all steps in fusion pore development, further elucidate the precise regulatory roles of SNARE accessory proteins, and deepen our understanding of neurotransmission as a whole.

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2.7 Figures

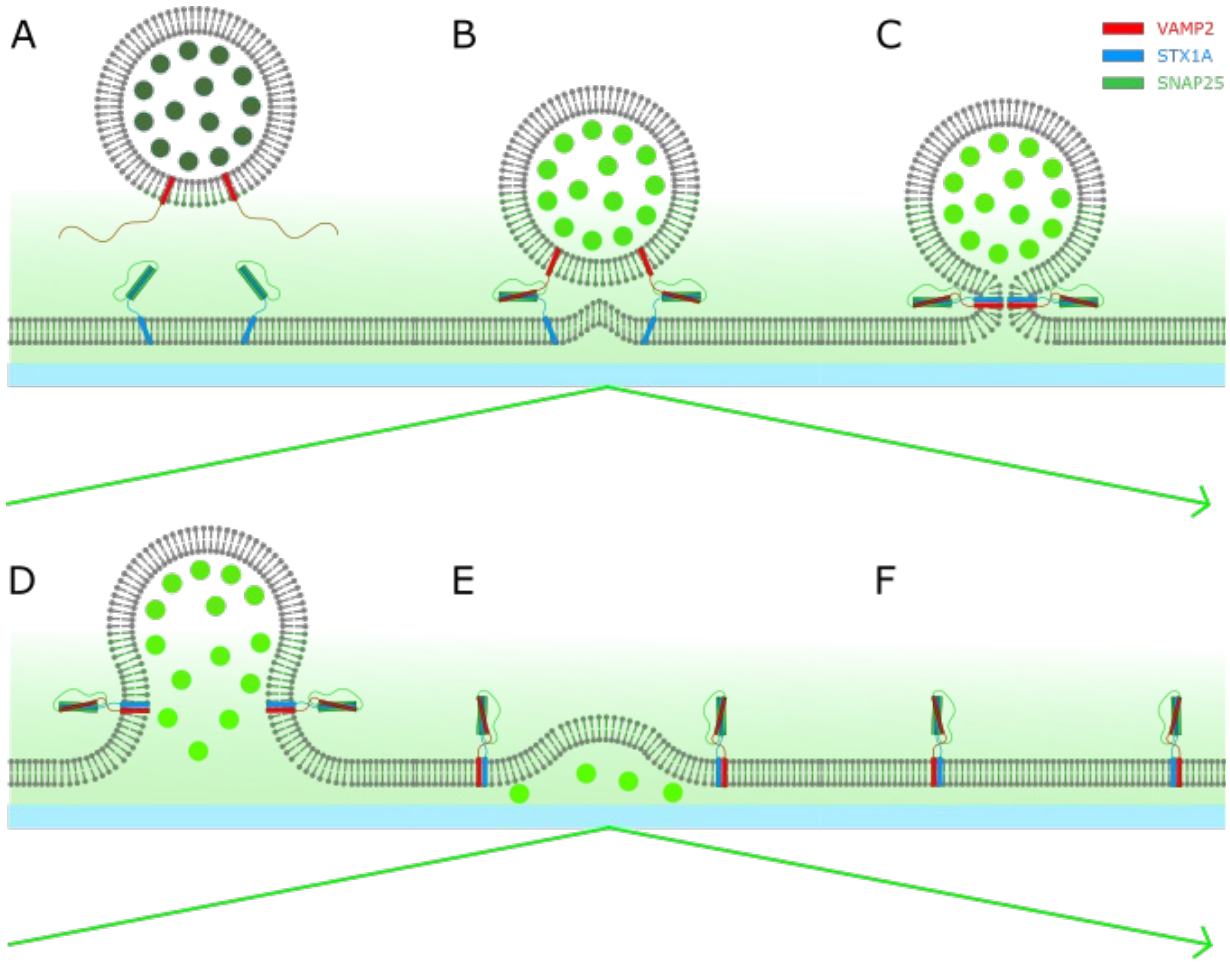

Figure 1. Schematic diagram of single vesicle-to-suspended bilayer content release assay. **(A)** V-vesicles containing fluorescent SRB-dextran content dye are injected into the flow cell. The distance of the vesicles in the flow cell from the surface of the glass slide prior to docking prevents excitation of the dye, limiting background noise. **(B)** Vesicle docks to the bilayer as v-SNAREs and t-SNAREs interact. Increased proximity to the slide permits excitation of vesicle content by an evanescent field generated using a TIRF laser. **(C)** Lipid merger and opening of the fusion pore. Initial fusion pore is too small to release 10kDa SRB-dextran content dye, allowing detection of pore expansion. **(D)** Expansion of the fusion pore permits release of content dye. The increased intensity of the evanescent field nearer to the slide may result in a brief intensity spike, followed by a decline due to lower remaining content. Fusion pore may contract to earlier steps, in which case it will appear as a dimmer docked vesicle after dispersal of the released content, or it may further expand. **(E)** Further expansion of the fusion pore results in full release and dispersal of fluorescent content dye. **(F)** Full fusion as the vesicle merges completely with the suspended bilayer.

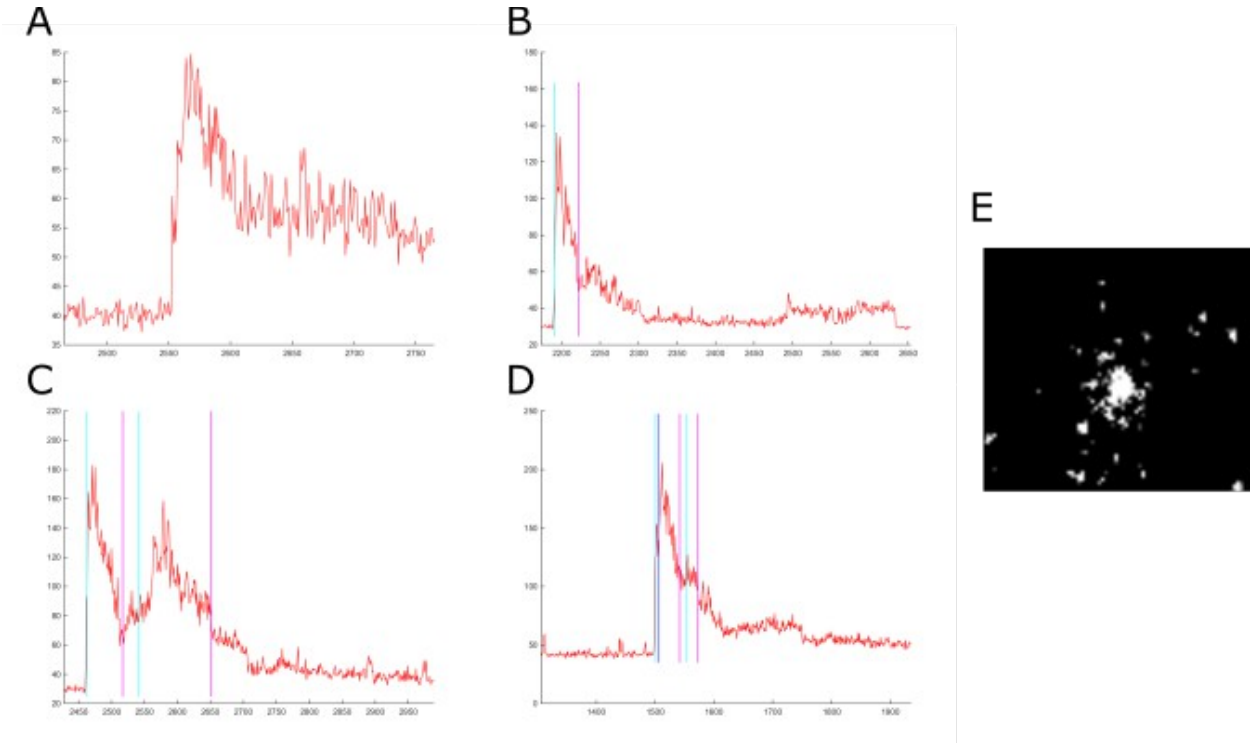

Figure 2. Representational fluorescence intensity traces of the SNARE-only content release system. **(A)** A trace taken from a docked vesicle without any visible release of SRB-dextran. Initial ntensity changes are likely due to changes in distance from the slide. **(B)** Trace taken from a vesicle with a single observed content release event. The cyan line indicates the start of release, while the magenta line indicates the end. **(C)** A trace taken from a vesicle with two distinct observed release events. **(D)** A trace taken from a vesicle with three observed release events. For traces (B, C, D), sharp drops in intensity after end of release events without accompanying release of their own may be due to disassembly of some formed SNARE-complexes, resulting in partial retreat of the vesicle from the bilayer. **(E)** TIRF microscope image of a vesicle releasing SRB-dextran content dye. The can be seen diffusing away from the vesicle.

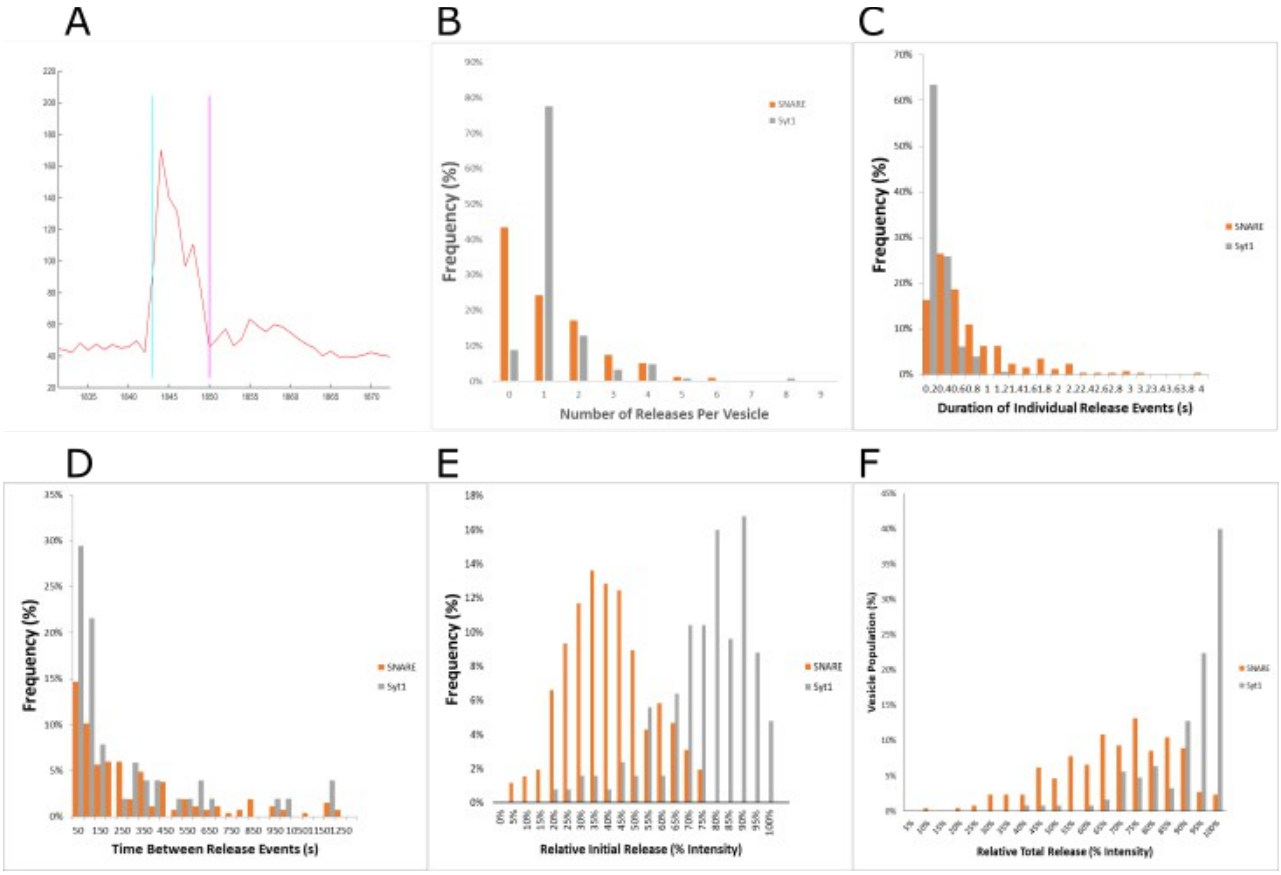

Figure 3. Comparison of pore dynamics in SNARE-only and SNARE-plus-Syt1 systems. **(A)** Representational trace of SNARE-plus-Syt1 vesicle in the presence of 500 μM Ca^{2+}. Dye fully diffuses away in less than 10 frames. **(B)** Frequency in number of individual observed release events per vesicle. Orange is the SNARE-only system, grey is the SNARE-plus-Syt1 system. **(C)** Duration of individual release events. Events are grouped in 200 ms intervals. **(D)** Time between individual release events on an individual vesicle. Delays in release are grouped into 50 ms intervals. **(E)** Percent of content released during the first release event for a given vesicle in 5% intervals. **(F)** Total apparent content released from individual vesicles after the end of all release from the vesicle.

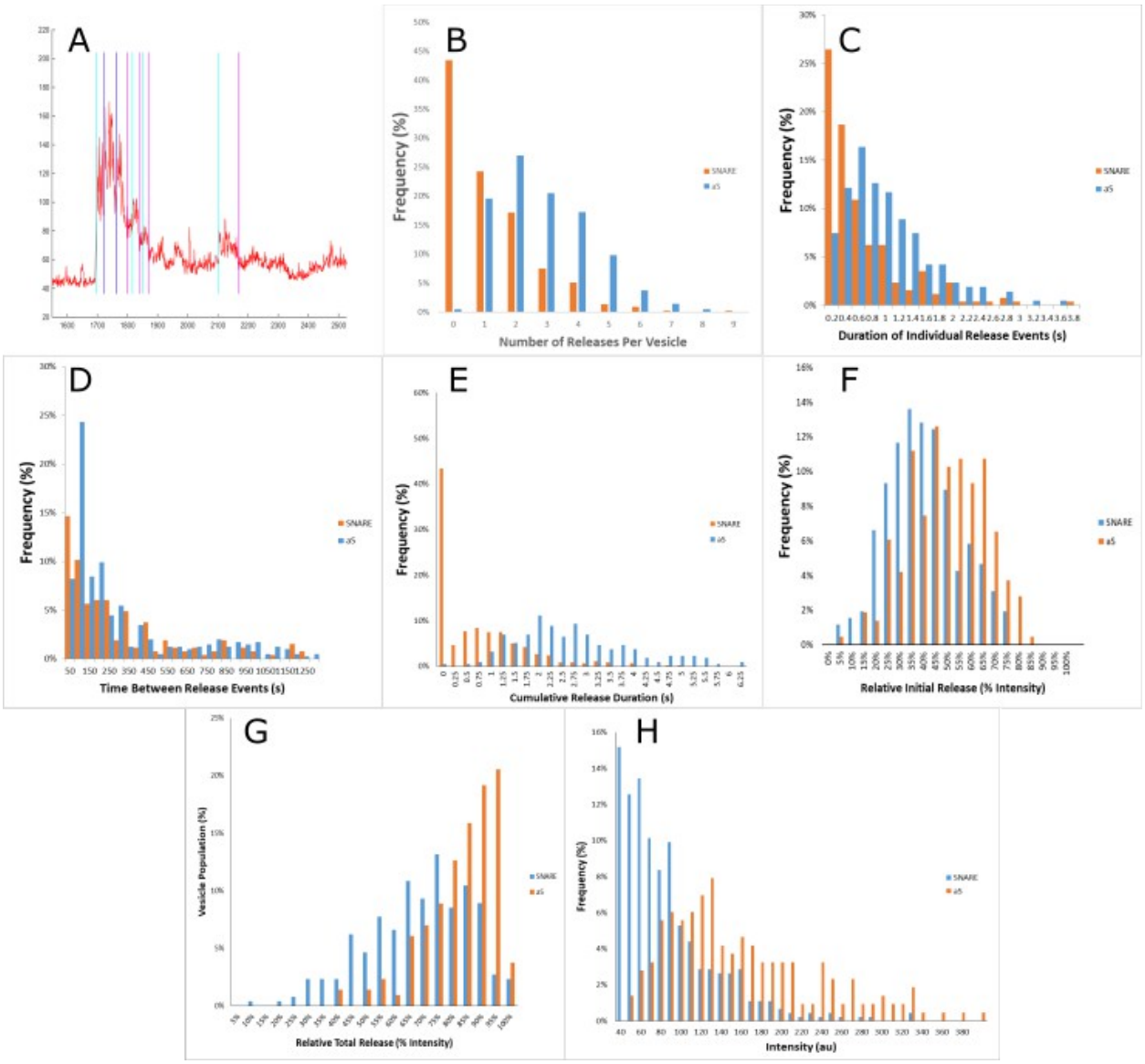

Figure 4. Comparison of pore dynamics in SNARE-only and SNARE-plus-αS systems. **(A)** Representational trace of SNARE-plus-αS vesicle. Vesicles in this system demonstrate an increase in average number of release events. **(B)** Frequency in number of individual observed release events per vesicle. Orange is the SNARE-only system, blue is the SNARE-plus-αS system. **(C)** Duration of individual release events. Events are grouped in 200 ms intervals. **(D)** Time between individual release events on an individual vesicle. Delays in release are grouped into 50 ms intervals. **(E)** Cumulative release duration from all events in individual vesicles. Total release times are grouped in 250 ms intervals. **(F)** Percent of content released during the first release event for a given vesicle in 5% intervals. **(G)** Total apparent content released from individual vesicles after the end of all release from the vesicle. **(H)** Frequency of observed maximum intensities in docked vesicles. Intensities are grouped in 10 intensity unit intervals.

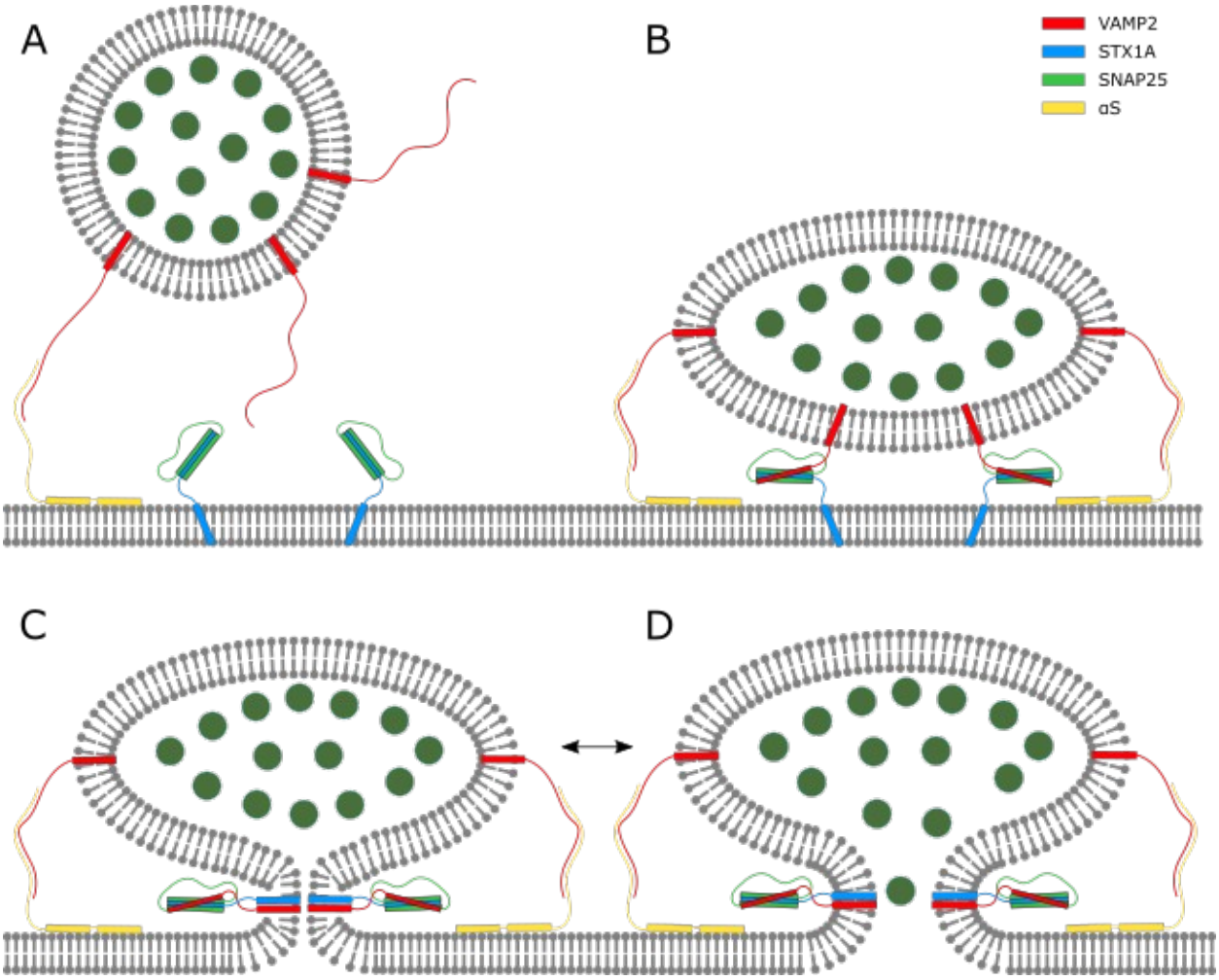

Figure 5. Hypothesized model for αS promotion of pore fluctuation. **(A)** The N-terminal region of αS binds to the pre-synaptic plasma membrane and interacts with VAMP2 on a nearby v-vesicle via its unstructured C-terminal region. This allows it to serve as a cross-bridge, drawing the vesicle to the bilayer and promoting docking. **(B)** The vesicle docks to the plasma membrane as αS draws it close enough for the v-SNAREs and t-SNAREs to interact. Additional membrane-bound αS interacts with other non-complexed VAMP2 on the vesicle, holding it more tightly to the plasma membrane and potentially stretching the vesicle. **(C and D)** The VAMP2-αS interaction continues to hold the vesicle in place against the membrane as the pore opens and fluctuates in size, while preventing retreat of the vesicle from the membrane. Other accessory proteins may interact with the SNARE complex here to further promote pore dilation. VAMP2 is shown in red, STX1A is shown in blue, SNAP25 is shown in green, and αS is shown in yellow.

CHAPTER 3

SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

SNARE-mediated vesicle fusion has long been a major topic of interest for its role in neurotransmission. While great strides have been made in exploring the overall process, elucidating individual steps and the regulatory roles of accessory proteins within them has historically been more difficult. This was particularly true for studies probing the dynamics of the fusion pore, given the limitations of older *in vitro* vesicle fusion assays. Until now, a lack of physiologically relevant systems permitting researchers to observe the evolution of the fusion pore in real time has been a major barrier to progress in this area.

In this study, we have demonstrated a new, robust *in vitro* method capable of overcoming these past issues. Using our TIRF single vesicle-to-suspended bilayer content release assay, we were able to observe the expansion and contraction of the SNARE-mediated vesicle fusion pore on a millisecond timescale. Notably, the results from this initial study demonstrate that the SNARE complex, while capable of driving the initial opening of the fusion pore, is unable to push the vesicle towards full or near-full fusion on its own. The support of accessory proteins, like the Ca^{2+}-sensor Syt1, is necessary to promote larger pore opening. This may be particularly relevant during the release of larger molecules like neuropeptides, which would have more difficulty than small molecule neurotransmitters in escaping from a small fusion pore of the kind formed by the SNARE complex alone.

Furthermore, the single vesicle-to-suspended bilayer content release assay permitted the observation of fluctuations in the size of the fusion pore. Multiple distinct release events from individual vesicles strongly suggest that the fusion pore goes through multiple stages of dilation

and contraction after it is formed, and that this tendency increases when an accessory protein like αS serves to stabilize the vesicle closer on the bilayer. The role of this fluctuation in the overall fusion process is unclear. However, it raises the possibility that other accessory proteins may promote or impede fusion through inhibition of reversible changes in pore size, suggesting a potential avenue for future exploration.

The single vesicle-to-suspended bilayer content release assay presents many new opportunities for the study of SNARE-mediated fusion. Our preliminary studies already present fascinating implications for the evolution of the fusion pore, and this could only benefit from more in-depth work using faster imaging instrumentation capable of directly detecting the diffusion of smaller dye molecules, permitting more flexibility in the detection of subtle changes in pore size. Even without this alteration, it permits studies dedicated to the detailed investigation of accessory proteins and their regulatory influence on fusion pore dilation. The continued pursuit of this work will, using our method, lead to a greater understanding of the mechanisms of neurotransmission and potentially provide a fascinating insight into the life cycles of synaptic vesicles.

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